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EFFECTS OF COLD STRATIFICATION AND GA_3 ON GERMINATION OF *ARBUTUS UNEDO* SEEDS OF THREE PROVENANCES

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Abstract

Background: *Arbutus unedo* is a valuable Mediterranean shrub as an ornamental plant as well as fruit tree. Fresh fruits of *A. unedo* are a good source of antioxidants, of vitamins C, E and carotenoids and also are characterized by the high content of mineral elements.
Materials and Methods: The effects of gibberellic acid (GA_3) and cold stratification (CS) on seed germination performance were investigated in *A. unedo* seeds collected from three provenances in the Northern part of Greece. Seeds of each provenance were soaked in solutions of GA_3 (500, 1000 or 2000 ppm) for 24 h and subsequently were subjected to CS at 3 – 5ºC for 0, 1, 2, and 3 months.
Results: Non-stratified seeds of the three *A. unedo* provenances which were not treated with GA_3 solutions exhibited very low germination. However, seed germination was significantly improved after a one-month period of CS. Similarly, the non-stratified seeds of all three provenances became non-dormant after the treatment with 2000 ppm GA_3 and they germinated at high percentages. However, in untreated seeds with GA_3, after a one-month CS period the seeds of the Pieria provenance exhibited higher germination percentage than that of Rodopi provenance seeds. Furthermore, in non-stratified seeds, the Pieria provenance seeds treated with GA_3 germinated at higher percentages and more rapidly than those of the other two provenances.
Conclusion: The results indicated that untreated seeds exhibited very low germination at 20/25ºC. However, in all three provenances seed germinability was significantly improved by a one-month period of CS or treatment of seeds with 2000 ppm GA_3. Furthermore, there was a considerable variability among seed provenances in response to the treatments which were applied.

Key words: pre-germination treatment, seed dormancy, strawberry tree, Mediterranean species.

Introduction

The genus *Arbutus* L. (Ericaceae) includes about 12 to 20 species (depending on the author) distributed from the West coast of North America through Mexico and Central America, Western Europe, the Mediterranean region, Northern Africa and parts of the Middle East (Hileman et al., 2001; Torres et al., 2002). According to Hileman et al. (2001), three species of genus *Arbutus* are distributed in the Mediterranean region: *A. unedo* L., *A. andrachne* L. and their hybrid *A.* x *andrachnoides* Link. *Arbutus unedo* is an evergreen species, which usually exists as a shrub of a height up to 2 – 3 m or sometimes a small tree up to 10 - 12 m. In Greece, it is one of the most important components of maquis communities (Boratynski et al., 1992). *Arbutus unedo* is also a valuable species as an ornamental plant due to its attractive white flowers which appear simultaneously with the orange-red fruits in the fall and winter (Celikel et al., 2008) as well as fruit tree. Fresh fruits of *A. unedo* are a good source of antioxidants, of vitamins C, E and carotenoids (Alarcao-E-Silva et al., 2001; Pallauf et al., 2008) and also are characterized by the high content of mineral elements (Ca, K, Mg, Na and P) (Ozcan and Haciseferogullari, 2007). Furthermore, the flowers of *A. unedo* are a significant source of nectar for bees (Soro and Paxton, 1999; Dalla Serra et al., 1999). Apart from the economic value of non-timber forest products, the ability of species to grow in dry areas (Hileman et al., 2001) and to vigorously resprout after fire (Espelta et al., 2012) makes it quite suitable for reforestation programs in Mediterranean regions.

Considering the above, in order to introduce the specific species in reforestation programmes, an easy propagation method has to be developed. For many species, propagation from seeds is the most common and the cheapest method used in nurseries (Macdonald, 2006). As a further benefit, the genetic diversity is promoted by propagation from seeds. However, a major constraint to the sexual propagation of many species is the poor germination of their seeds. This is possibly due to low viability, although it is frequently due to seed dormancy (Mackay et al., 2002), which is a physiological state during which a viable seed fails to germinate even when the environment is favorable to germination (Macdonald, 2006). However, in the relevant literature there is conflicting information regarding the dormancy of *A. unedo* seeds. Several references report that *A. unedo* seeds show dormancy and that cold stratification is used to overcome it (Tilki, 2004; Smiris et al., 2006; Demirsoy et al., 2010; Ertekin and Kirdar, 2010). Furthermore, the treatment of seeds with GA_3 is effective to break dormancy and increase the germination in *A. unedo* and *A. andrachne* (Karam and Al-Salem, 2001; Tilki, 2004; Demirsoy et al., 2010). In contrast, Ricardo and Veloso (1987), Mesleard and Lepart (1991) and Bertsouklis and Papafotiou (2013) stated that non-stratified seeds of *A. unedo* germinate at high

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percentage when they are placed at alternating temperatures of 15 and 20ºC or at a constant temperature of 15ºC or less. Although seed dormancy has been studied by numerous researchers, no attention has been given on the variation in germination characteristics among populations of the species. Germination characteristics of seeds collected from different populations can vary in degree of dormancy, germination rates, environmental conditions (temperature, substrate moisture, pH, calcium, and salinity) required for germination as well as in the amount of cold stratification required to break dormancy (Baskin and Baskin, 1998). Applying the recommended germination protocol may incur the risk of poor germination, resulting in increased production cost when the variability in seed germination requirements among provenances is not taken into account.

The present study aims to evaluate the effect of gibberellic acid (GA_3) and cold stratification treatments (and their combinations) on germination of *A. unedo* seeds and also to reveal the existence of variability in germination of three *A. unedo* provenances by evaluating the germination characteristics (germination percentage and rate) of seeds subjected to cold stratification and gibberellic acid (GA_3) treatments (and their combinations).

Materials and Methods

Seed collection

Mature fruits of *A. unedo* were collected from shrubs growing in natural habitats from three provenances. In particular, fruits were collected from three prefectures located in the Northern part of Greece (Rodopi, Chalkidiki, Pieria) (Table 1). After collection, the fruits were pulped by hand and the separation of seeds from pulp was achieved using sieves and running water. In addition, floated seeds were removed during cleaning, and subsequently, the clean seeds were spread out on filter papers in laboratory conditions and left to dry for a week. After drying, the moisture content of seeds as well as the number of seeds per grammar were calculated for each provenance (Table 1) according to the rules of ISTA (1999) and then the seeds were stored in glass containers in the refrigerator (3 - 5ºC) until they were used in the experiments.

Seed treatment

Germination experiments were started the following February and conducted in the laboratory of Silviculture, Department of Forestry and Management of the Environment and Natural Resources, Democritus University of Thrace.
For each provenance, an experiment was carried out to determine the effects of gibberellic acid (GA_3), cold stratification (CS) and combination of GA_3 with CS on seed germination. Seeds of each provenance were soaked in solutions of GA_3 (two volumes of GA_3 Solution for each volume of seeds) for 24hours at room conditions. The concentrations of GA_3 solutions were 500, 1000 and 2000 ppm. Subsequently, the treated seeds were placed between two moist layers of filter paper in plastic containers and given CS at 3 - 5ºC for 0, 1, 2, and 3 months. In addition, seeds from each provenance were soaked in distilled water for 24 hours (control) and then were subjected to CS for 0, 1, 2, or 3 months. For each provenance there were four plastic containers (3 of them corresponded to the three concentrations of GA_3 and one to control seeds). During stratification, filter papers moisture was checked periodically and water was added as needed.

Germination test

For each provenance, at the end of each CS period, a random sample of 120 seeds were taken out from each plastic container and randomly placed in 4 plastic Petri dishes (30 seeds per Petri dish). For each treatment, there were 4 replications of 30 seeds. Seeds were placed on two layers of filter paper moistened with distilled water in 9-cm plastic Petri dishes. The Petri dishes were randomly arranged on the shelves of the growth chamber and were watered with distilled water, as necessary. The temperature in the growth chamber was set at 20ºC for a 16-hour dark period and 25ºC for a 8-hour light period (Provided by cool white fluorescent tubes with an intensity of 75 µmol-m-2-s-1. Germinated seeds were counted once a week for a period of 6 weeks. A seed was considered as germinated when the radicle had emerged through the seed coat. Finally, for each treatment of each provenance the germination percentage (GP) and the mean germination time (MGT) were calculated as the average of the 4 replications. The MGT was calculated for each replication per treatment according to the following equation:

$$MGT = \Sigma(Dn)/\Sigma n$$

where n is the number of seeds which germinated on day D and D is the number of days counted from the beginning of the test (Hartmann et al., 1997).

Statistical analysis

For each provenance, a completely randomised experimental design was used. It is worth noting that, in all three provenances a CS period longer than one month was not used as, at the many germinated seeds appeared during the two-months period of CS. In each provenance, the germination percentage data, which were firstly arc-sine square-root transformed (Snedecor and Cochran, 1980), as well as MGT data were analysed by one-way ANOVA. Furthermore, in each treatment (combinations of GA_3 and CS treatment) the germination percentage data as well as MGT data of the three provenances were analysed by one-way ANOVA. The means were compared using the Duncan test (Klockars and Sax, 1986). All statistical analyses were carried out using SPSS 21.0 (SPSS, Inc., USA).

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Results

The number of seeds per gram was 510 at 5.07% moisture content for Rodopi provenance, 441 at 5.51% for Chalkidiki provenance and 405 at 5.05% for Pieria provenance (Table 1).

Table 1: Provenances of the collected *A. unedo* seeds.

Provenances	Altitude (m a.s.l)	Latitude	Longitude	Collection date	Number of seeds/g	Moisture content (%)
Rodopi	255	41º08′38′′N	25º15′35′′E	25/11/2013	510	5.07
Chalkidiki	15	40º35′13′′N	23º47′43′′E	11/12/2013	441	5.51
Pieria	500	40º11′26′′N	22º19′25′′E	7/12/2013	405	5.05

In all three provenances, the analyses of variance indicated that there were significant differences in germination percentages as well as in MGT (a=0.05) among the combinations of GA_3 concentrations and CS periods (Tables 2 and 3).

Table 2: Germination percentages of *A. unedo* seeds of the three provenances, after various combinations of GA_3 and cold stratification treatments

GA_3 (ppm)	Cold stratification (months)	Germination percentage (%, ± S.D.)		
		Rodopi	Chalkidiki	Pieria
0	0	0.83 f[1] **b**[2] ± 1.67	3.33 e **ab** ± 4.71	7.50 d **a** ± 4.19
	1	81.67 bc **b** ± 1.92	88.34 b **ab** ± 1.92	90.83 a **a** ± 5.69
500	0	49.17 e **b** ± 4.19	40.00 d **c** ± 4.71	79.17 c **a** ± 5.00
	1	91.67 a **a** ± 1.92	94.17 a **a** ± 5.00	81.67 bc **b** ± 6.38
1000	0	68.33 d **b** ± 6.39	67.50 c **b** ± 1.67	80.84 c **a** ± 6.31
	1	88.34 a **b** ± 3.33	95.84 a **a** ± 1.67	83.34 abc **b** ± 6.09
2000	0	80.00 c **b** ± 4.71	80.83 b **b** ± 5.69	90.00 ab **a** ± 6.09
	1	86.67 ab **b** ± 2.72	97.50 a **a** ± 1.66	85.83 abc **b** ± 3.19

[1] In a column, percentages are statistically different at $p < 0.05$, when they don't share a common letter (letters in normal font). [2] In a row, percentages are statistically different at $p < 0.05$, when they don't share a common letter (letters in bold font). The comparisons were made using Duncan test.

Table 3: Mean Germination Time of *A. unedo* seeds of the three provenances, after various combinations of GA_3 and cold stratification treatments

GA_3 (ppm)	Cold stratification (months)	Mean Germination Time (days, ± S.D.)		
		Rodopi	Chalkidiki	Pieria
0	0	*	*	14.00 b ± 4.95
	1	8.71 a[1] **a**[2] ± 0.51	8.84 a **a** ± 0.55	8.24 a **a** ± 0.54
500	0	22.81 c **b** ± 1.59	20.98 b **b** ± 1.94	15.57 b **a** ± 0.53
	1	9.47 a **a** ± 0.80	9.35 a **a** ± 0.55	9.55 a **a** ± 0.81
1000	0	20.29 b **b** ± 0.65	19.98 b **b** ± 2.19	16.03 b **a** ± 0.81

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	1	8.13 a **a** ± 0.72	8.27 a **a** ± 0.63	8.20 a **a** ± 0.57
2000	0	21.16 b **b** ± 1.57	20.39 b **b** ± 1.86	16.12 b **a** ± 1.08
	1	9.43 a **a** ± 0.70	8.56 a **a** ± 0.80	8.65 a **a** ± 0.83

* MGT was not calculated because in one of the four replications, no seed germinated. [1] In a column, means are statistically different at $p < 0.05$, when they don't share a common letter (letters in normal font). [2] In a row, means are statistically different at $p < 0.05$, when they don't share a common letter (letters in bold font). The comparisons were made using Duncan test.

In all three provenances, non-stratified seeds of *A. unedo* which were not treated with GA_3 solutions exhibited very low germination (0.83 – 7.50%). In Rodopi and Chalkidiki provenances, regardless of GA_3 treatment, seeds stratified for 1 month exhibited higher ($p < 0.05$) GPs than that of non-stratified seeds. Whereas, in Pieria provenance, in seeds only treated with GA_3 solutions, there were no significant differences ($p > 0.05$) in GPs between non-stratified seeds and seeds stratified for 1 month. In all three provenances, GA_3 application significantly improved the germination of non-stratified seeds. Furthermore, in Rodopi and Chalkidiki provenances increasing the concentration of GA_3 resulted in a significant increase ($p < 0.05$) in GPs of non-stratified seeds. In Pieria provenance, non-stratified seeds which were treated with 2000 ppm exhibited the highest ($p < 0.05$) GP, whereas there were no significant differences ($p > 0.05$) in GPs between seeds treated with 500 and 1000 ppm. After a one-month period of CS, the germination percentage of seeds of Rodopi provenance which had been treated with 500 and 1000 ppm GA_3 was higher ($p < 0.05$) than that of the seeds which had not been treated with GA_3, whereas in Chalkidiki provenance the seeds treated with GA_3, regardless of concentration, exhibited higher ($p < 0.05$) GPs than those not treated with GA_3. In contrast, after a one-month CS period of Pieria provenance seeds, untreated seeds with GA_3 exhibited higher ($p < 0.05$) GP than those treated with 500 ppm GA_3, whereas there were no significant differences with seeds treated with 1000 or 2000 ppm.

In all three provenances, the seeds stratified for 1 month, regardless of GA_3 treatment and concentration, exhibited the lowest ($p < 0.05$) MGT. Only in Rodopi provenance the MGT of non-stratified seeds was affected by concentration of GA_3. Seeds treated with 1000 and 2000 ppm exhibited lower ($p < 0.05$) MGT than those treated with 500 ppm.

Statistical analysis also revealed significant differences in GPs and MGT among the three provenances (Tables 2 and 3). In non-stratified seeds which were treated with GA_3, regardless of concentration, Pieria provenance exhibited the highest ($p < 0.05$) GPs and the lowest ($p < 0.05$) MGT. After 1 month of CS, in seeds which were not treated with GA_3, Pieria provenance exhibited higher ($p < 0.05$) GP than the Rodopi provenance, whereas in seeds treated with 500ppm. Pieria provenance exhibited lower ($p < 0.05$) GP than the other two provenances. In seeds treated with 1000 and 2000 ppm which were stratified for 1 month, Chalkidiki provenance exhibited the highest ($p < 0.05$) GPs.

As regards MGT, in seeds stratified for 1 month, regardless of GA_3 treatment and concentration, no significant differences ($p > 0.05$) were observed among the three provenances.

Discussion

Non-stratified seeds of the three *A. unedo* provenances, which were not treated with GA_3 solutions, exhibited very low germination (0.83 – 7.50%). The same results were also provided by Tilki (2004), Demirsoy et al. (2010), Ertekin and Kirdar (2010), who found very low germination percentages. In the present study, as in the studies of the above researchers, the seeds germinated in a temperature range between 18 and 25°C. Ricardo and Veloso (1987) and Bertsouklis and Papafotiou (2013) found that non-stratified seeds of *A. unedo* germinated at percentages about 40 and 30%, respectively when they were placed in constant temperature at 20ºC, while in both studies the seeds failed to germinate at 25ºC. However, in their experiment, non-stratified seeds germinated at very high percentage (> 80%) when they were placed at temperature equal or lower than 15ºC. Perhaps, *A. unedo* seeds are conditionally dormant and they germinate over a narrow range of low temperatures (10 – 15ºC).

For all three provenances, seed germination was significantly improved by cold stratification treatment. In untreated seeds with GA_3, after a one-month period of CS, the germination percentages of seeds of all three provenances were very high (> 80%). Although, Tilki (2004) reported that a six-week period of CS resulted in 50% germination and increasing CS duration to 9 or 12 weeks increased germination of *A. unedo* seeds (86 and 84%, respectively). Furthermore, Smiris et al. (2006) referred that increasing CS duration from 1 to 2 and 3 months increased germination of *A. unedo* seeds from 0 to 26 and 48%, respectively. In the present study, the response to longer period of CS (2 months) was similar for all three provenances, many seeds germinated during the moist stratification at 3 – 5ºC. After a CS treatment, seeds came out of dormancy and they germinated over a wide range of temperatures. According to Baskin and Baskin (1998) non-dormant seeds germinate over a wider range of conditions than do conditionally dormant seeds. Similarly, after the treatment with 2000 ppm GA_3 the seeds of all three provenances became non-dormant and they germinated at high percentages (≥ 80%) at 20/25ºC. Possibly, the treatment of seeds with a high concentration solution of GA_3 counteracted the inhibitory effect of high temperatures on germination. According to Ricardo and Veloso (1987), *A. unedo* seeds treated only with 500 ppm GA_3 germinated at percentages about 90% and 50% at 20ºC and 25ºC, respectively. Seeds of *Kalidium gracile*, which exhibit primary conditional dormancy, become non-dormant by a short period of CS or by GA_3 treatment (Cao et al., 2014). Furthermore, the CS, as well as the exogenous GA_3 application, has been reported to be effective in breaking dormancy in the seeds of *Arbutus* species (Karam and Al-Salem, 2001; Tilki, 2004; Smiris et al., 2006; Demirsoy et al., 2010; Ertekin and Kirdar, 2010). As far as the germination rate is concerned, the CS for 1 month of seeds,

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regardless of GA_3 treatment, resulted in the most rapid germination (the lowest values in MGT) for all three provenances. In practice, apart from high seed germination, uniform and rapid seed germination is also significant in order to avoid environmental hazards in the nursery. Thus, for propagation purposes a CS period of seeds is recommended as an effective treatment for maximum, rapid and uniform germination of *A. unedo* seeds over a wide range of temperatures.

The CS, as well as the GA_3 application, significantly improved the germination of the three seed provenances of *A. unedo*. However, there was a considerable variability among seed provenances in response to treatments which were applied. In untreated seeds with GA_3, after a one-month CS period the seeds of the Pieria provenance exhibited higher GP than those of Rodopi provenance seeds. Germination characteristics of seeds collected in various locations can vary in degree of dormancy, as reflected by GPs of fresh seeds (Baskin and Baskin 1998). As mentioned, in all three provenances a CS period longer than one month was not used as germinated seeds appeared at the end of the two-month period of CS. The germination percentage of Rodopi provenance seeds may be higher if a longer period of CS is used, for example one and half months. The effects of GA_3 application on seed germination varied among the three provenances. The results indicated that in non-stratified seeds, the Pieria provenance seeds treated with GA_3, regardless of concentration, germinated at higher percentages and more rapidly (lower values in MGT) than those of the other two provenances. The GA_3 application in seeds of Pieria provenance counteracted in greater degree the inhibitory effect of high temperatures on germination, than in seeds of the other two provenances. Whereas, in seeds stratified for 1 month, the Pieria provenance seeds treated with GA_3, regardless of concentration, exhibited lower GP than those of Chalkidiki provenance. Taking into account the results of the present and previous studies, variability in germination requirements due to provenance of *A. unedo* seeds is observed. Smiris et al. (2006) studying the germination of *A. unedo* seeds, which were collected in Western Greece, reported that the maximum percentage of germination (85.75%) was observed when the seeds were treated for 24 hours with 500 ppm GA_3 and then cold stratified for a period of 3 months. Furthermore, Bertsouklis and Papafotiou (2013), in *A. unedo* seeds collected in South Greece found that seeds stratified for 40 days germinated at about 80 and 15%, when they were placed in constant temperatures at 20 and 25ºC, respectively. This variability in germination of *A. unedo* seeds among provenances may reflect adaptations to differing environmental conditions. Similarly, Ricardo and Veloso (1987) observed variability in seed germination in high temperature among three provenances of *A. unedo* seeds from South Portugal.

Conclusions

Based on the results of the present research, it can be concluded that untreated seeds of the three *A. unedo* provenances exhibited very low germination at 20/25ºC. However, seed germination was significantly improved after a one-month period of CS. Similarly, the treatment of seeds only with 2000 ppm GA_3 was successfully overcome the dormancy in *A. unedo* seeds. There was a considerable variability among seed provenances in response to the treatments which were applied. In untreated seeds with GA_3, after a one-month stratification period the seeds of the Pieria provenance exhibited higher germination percentage than that of Rodopi provenance seeds. Furthermore, in non-stratified seeds, the Pieria provenance seeds treated with GA_3, regardless of concentration, germinated at higher percentages and more rapidly than those of the other two provenances.

In reforestation programs, emphasis is given in the use of local provenances as they have adapted to local environments. So, in an untested seed provenance of *A. unedo*, considering the variability in germination requirements among provenances of the species, in order to maximize seed-germination the best duration of CS and GA_3 concentration have to be determined first on a small sample before treating all the seed lot.

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